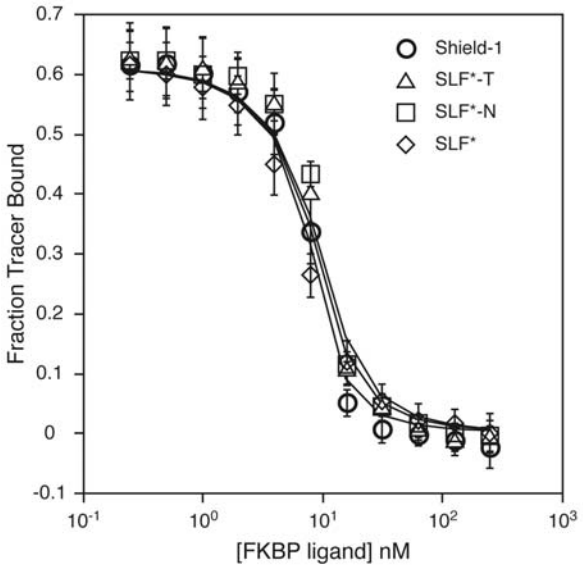
Supporting Information

A directed approach for engineering conditional protein stability using biologically silent small molecules

Lystranne A. Maynard-Smith*, Ling-chun Chen†, Laura A. Banaszynski*, A. G. Lisa Ooi†, and Thomas J. Wandless†‡

Departments of *Chemistry and †Chemical & Systems Biology, Stanford University
318 Campus Drive, Stanford, CA 94305

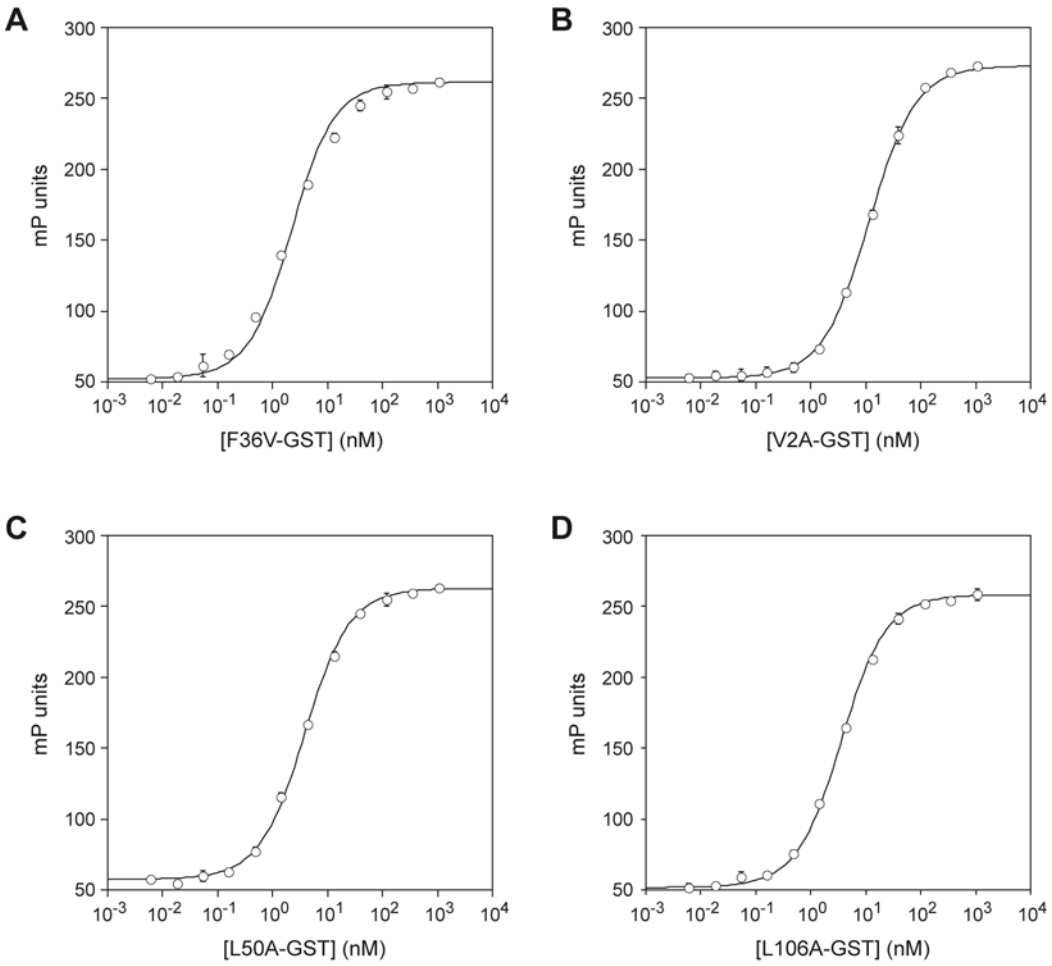
SI Figure 1



SLF* analog	K_D (nM)	R^2
Shield-1	0.4	0.983
SLF*-T	0.7	0.989
SLF*-N	0.9	0.986
SLF*	0.4	0.989

Figure 1. Competition Binding Assay between FKBP F36V and SLF* Analogs. Fluorescein-SLF (FL-SLF, 1 nM) and FKBP F36V (10 nM) were treated with varying concentrations of each SLF* analog (2-fold dilutions from 300 nM to 0.1 nM) and incubated at room temperature for 45 min. The plates were analyzed by fluorescence polarization, and the data were processed to determine fraction bound tracer at each competitor concentration. Data were then fit to determine the dissociation constant of each competitor as described (reference 1). Data are represented as the average of an experiment performed in quadruplicate ± SD.

SI Figure 2

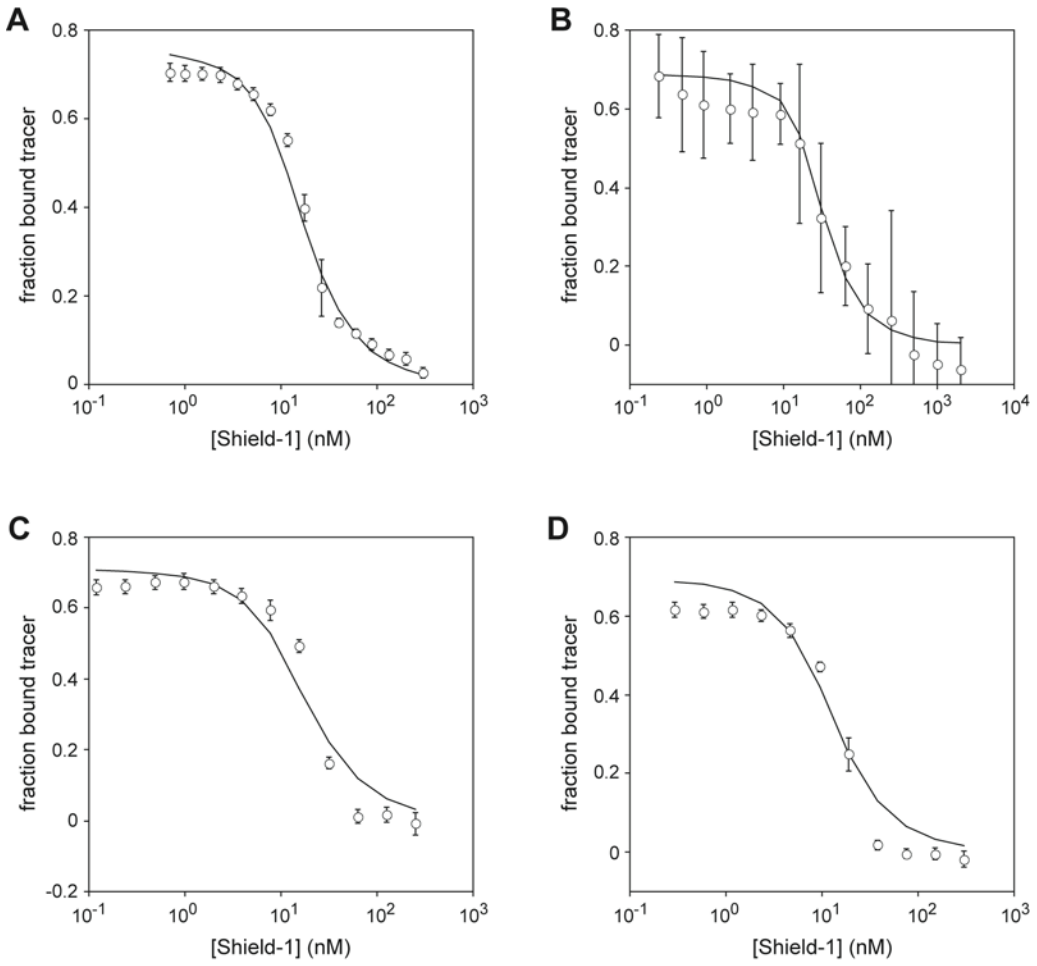


E

Protein	K_D (nM)
F36V GST	1.7 ± 0.2
V2A GST	11.0 ± 0.3
L50A GST	3.3 ± 0.1
L106A GST	3.1 ± 0.1

Figure 2. Saturation Binding Curves of FKBP-GST Fusion Proteins with FL-SLF. A fixed concentration of FL-SLF (1 nM) was incubated with various dilutions of the FKBP-GST fusion proteins in a 96-well plate. After 30 min incubation at room temperature, the plates were analyzed by fluorescence polarization. Each curve represents one independent experiment in quadruplicate wells. (A) F36V GST; K_D = 1.7 ± 0.2 nM. (B) V2A GST; K_D = 11.0 ± 0.3 nM. (C) L50A GST; K_D = 3.3 ± 0.1 nM. (D) L106A GST; K_D = 3.1 ± 0.1 nM. (E) Dissociation constants of FL-SLF tracer for FKBP-GST mutants.

SI Figure 3



E

Protein	K_D (nM)	R^2
F36V GST	2.0	0.987
V2A GST	4.0	0.972
L50A GST	3.0	0.956
L106A GST	2.0	0.958

Figure 3. Competition Binding Curves between FKBP-GST Proteins and Shield-1. The FL-SLF tracer (1 nM) and a fixed concentration of each of the fusion proteins were mixed with various dilutions of Shield-1 and incubated at room temperature for 45 min. The plates were then analyzed by fluorescence polarization, and the data were processed to determine the fraction of bound tracer at each competitor concentration. Data were then fitted for the K_D value of each fusion protein as described (reference 1). **(A)** [F36V-GST] = 10 nM; K_D = 2 nM. **(B)** [V2A-GST] = 25 nM; K_D = 4 nM. **(C)** [L50A-GST] = 8 nM; K_D = 3 nM. **(D)** [L106A-GST] = 7.5 nM; K_D = 2 nM. **(E)** Dissociation constants of Shield-1 for FKBP-GST fusion proteins.

SI Figure 4

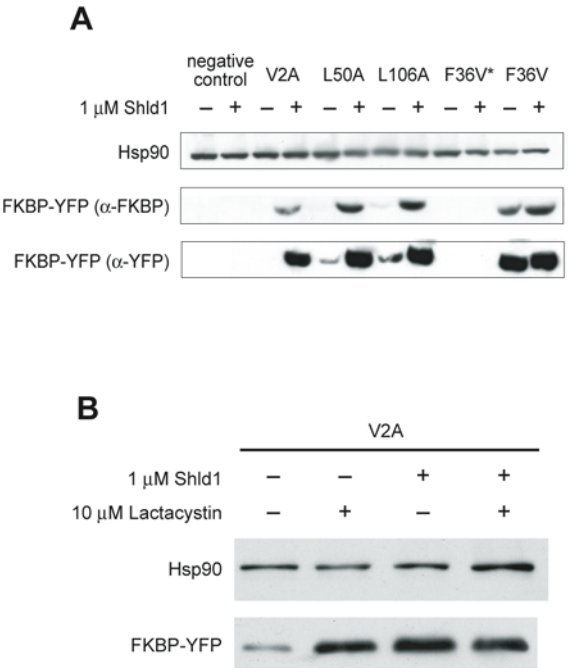


Figure 4. Degradation of FKBP-YFP fusions. **(A)** NIH3T3 cells stably expressing the indicated fusion proteins were mock-treated (–) or treated with 1 μM Shield-1 (+) for 24 hours. Cell lysates were resolved by SDS-PAGE and immunoblotted using an anti-FKBP antibody (top panel) and an anti-YFP antibody (lower panel). F36V* denotes the triple mutant (V2A/L50A/L106A) and Hsp90 was used as a loading control. **(B)** Degradation of FKBP-YFP fusion is mediated by the proteasome. NIH3T3 cells stably expressing the V2A-YFP fusion were treated with 1 μM Shield-1 for 24 hrs. Shield-1 was removed by washing the cells, which were then treated with 10 μM lactacystin in the presence and absence of Shield-1 for 4 hours. Immunoblotting was performed using an anti-YFP antibody.

SI Figure 5

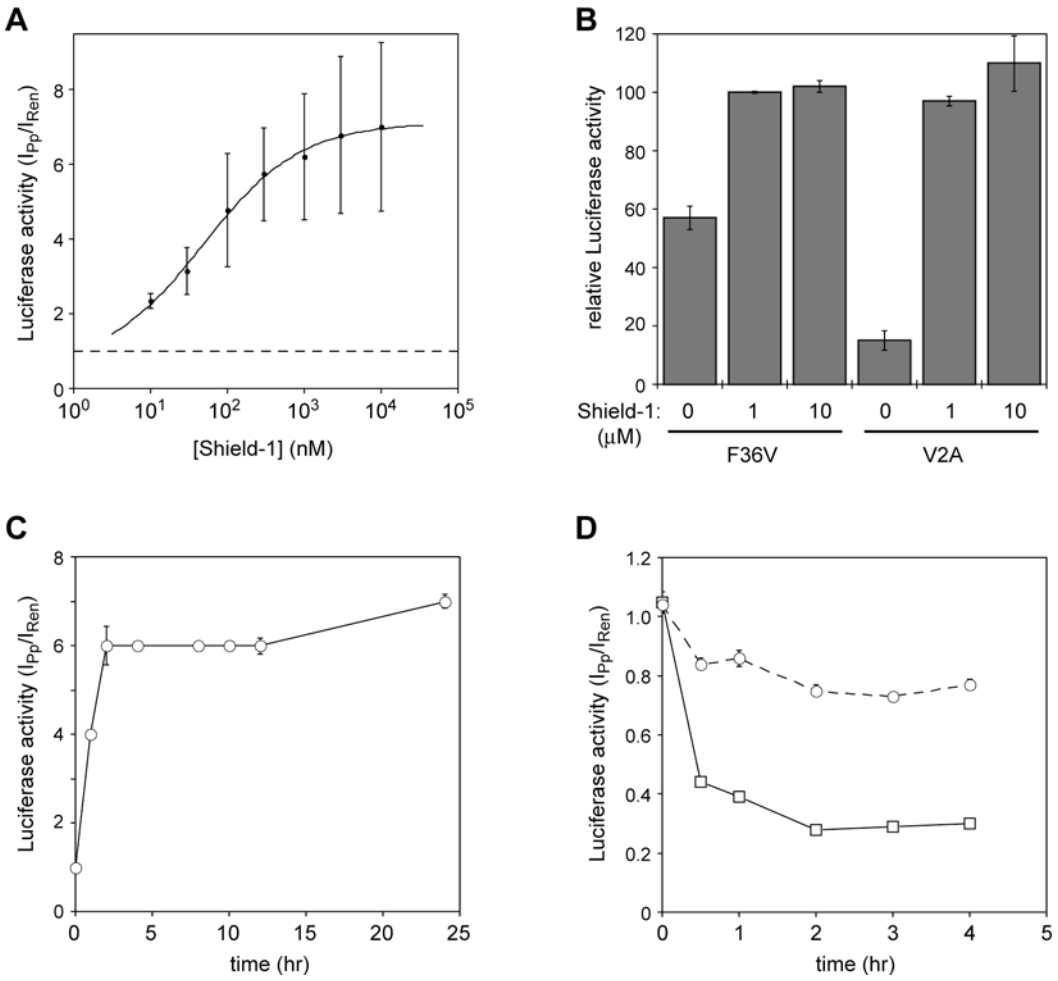
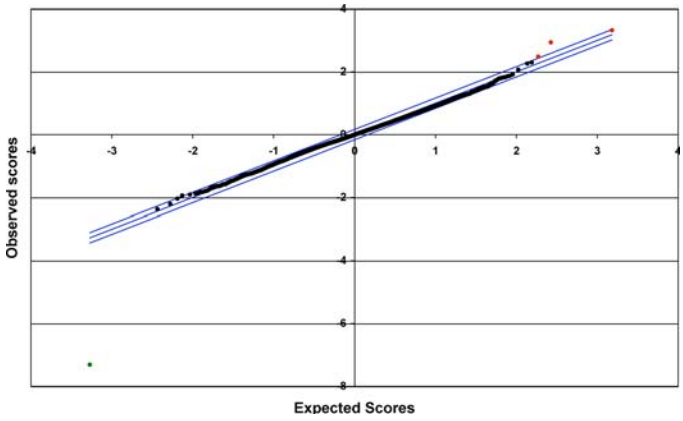


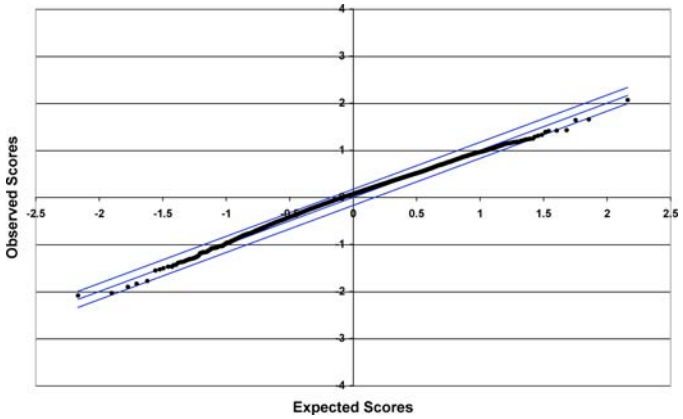
Figure 5. The FKBP V2A mutant destabilizes luciferase. Luminescence data are plotted as the ratio of *Photinus pyralis* (firefly) luciferase (Luc) to Renilla luciferase. **(A)** NIH3T3 cells stably expressing the FKBP V2A mutant fused to the N-terminus of the *Photinus pyralis* (firefly) luciferase were treated for 24 hours with 3-fold dilutions of Shield-1 (10 μM to 10 nM) and assayed using a dual luciferase reporter assay. The ratio of firefly/Renilla luciferase in the absence of Shield-1 (dotted line) is normalized to unity. Data are presented as the average mean luminescence intensity ± SEM relative to that of the maximum luminescence intensity observed. **(B)** F36V-Luc and V2A-Luc were mock-treated or treated with 1 or 10 μM Shield-1. Relative luminescence is plotted showing F36V-Luc and V2A-Luc. Data represent the average mean luciferase activity ± SEM relative to that of maximum luciferase activity observed in assay. Experiment was performed in triplicate. **(C)** NIH3T3 cells stably expressing V2A-Luc were treated with 1 μM Shield-1, and increases in luciferase activity were monitored over time. Data shown represent the mean luciferase activity ± SEM relative to mock-treated cells. Experiment was performed in triplicate. **(D)** NIH3T3 cells stably expressing V2A-Luc and F36V-Luc fusions were treated with 1 μM Shield-1 for 24 hours. The cells were then washed with media to remove Shield-1, and decreases in luciferase activity were monitored. Data represent the mean luciferase activity ± SEM relative to maximum activity for the individual mutant.

SI Figure 6

A. 1 μM Shield-1 treated versus mock-treated control



B. 100 nM Shield-1 treated versus mock-treated control



C. 10 nM Shield-1 treated versus mock-treated control

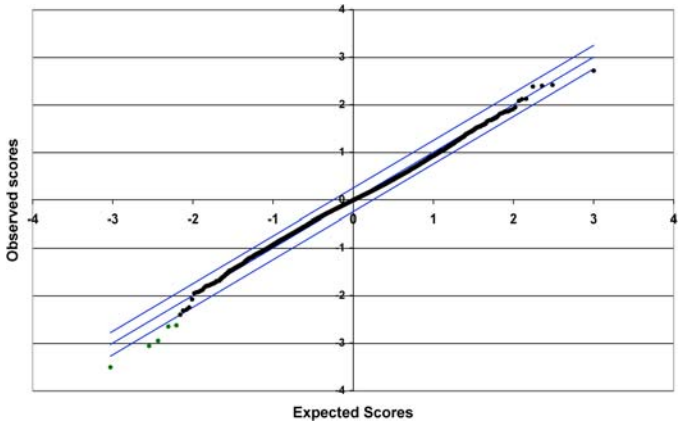


Figure 6. Significance Analysis of Microarrays (reference 2) was used to identify genes whose mRNA levels varied between mock-treated cells and cells treated with 1 μM Shield-1 (panel A), 100 nM Shield-1 (panel B), and 10 nM Shield-1(panel C). Decreases in mRNA levels upon Shield-1 treatment (relative to mock-treated control) are shown in green and increases are shown in red.

Experimental Details: Microarray analysis of NIH3T3 cells treated with Shield-1

NIH3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated donor bovine serum (Invitrogen), 2 mM glutamine, 100 U/mL penicillin, and 100 μg streptomycin. Populations of cells (10-cm plates, 10 mL media) were cultured for 24 hours in media containing 1 μM, 100 nM, or 10 nM Shield-1 or mock-treated with vehicle. Three independent cultures were treated for each of the four experimental groups. Total RNA was extracted from all twelve samples using the RNeasy kit (Qiagen) labeled, and hybridized to the Mouse Exonic Evidence Based Oligonucleotide (MEEBO) arrays. The oligonucleotide set consists of 38,784 70-mer probes that were designed using a transcriptome-based annotation of exonic structure for genomic loci (http://www.microarray.org/sfgf/meebo.do). The hybridizations were performed by Stanford Functional Genome Facility using its standard protocol. The complete Oligo Array Hybridization protocol can be found at http://www.microarray.org./sfgf/docView.do?type=2. In brief, thirty micrograms of total RNA was used for each labeling reaction using Superscript II reverse transcriptase (Invitrogen). Mouse RNA pooled from 11 cell lines (Stratagene) was used as reference in each hybridization. Two fluorescent dyes, Cy5-dUTP and Cy3-dUTP, were used to label the experimental cDNA and the Reference samples, respectively. After overnight hybridization at 65 ˚C, each microarray was washed and scanned using the GenePix 4000A microarray scanner (Axon Instruments). Twelve hybridizations were performed in total, corresponding to triplicates of the four experimental groups mentioned above.

Data selection and filtering

We selected the flagged array spots with a regression correlation of > 0.6 and with the fluorescence intensity greater than twice of the local background in either the Cy3 or the Cy5 channel. Only genes meeting the above criteria and that were available for greater than 80% of the arrays in each comparison were included for data analysis. The data were normalized by total intensity normalization, and sample/reference ratios were transformed to log(base 2) scale for data analysis.

Data analysis

To identify genes whose expression changed significantly after Shield-1 treatment at different concentrations, we used the unpaired two-class significant analysis of microarrays (reference 2). SAM computes a set of gene-specific *t*-tests and calculates a score for each gene based on its change in gene expression relative to the standard deviation in multiple samples. The *t*-statistic allows comparison at a chosen threshold value to determine if the expression of the gene was significant at this threshold. Each threshold value corresponds to a false discovery rate (FDR, the percentage of genes identified as "significant" by chance only), which was generated by randomly permutated data. A false discovery rate, q-value, was also given for each gene. The q-

value is like the familiar "p-value" adapted for the analysis of a large number of genes. Missing values was imputed using the average expression of the other two features for a particular gene.

Three sets of comparisons were made: (1) treatment with 10 nM Shield-1 versus mock-treated cells: (2) treatment with 100 nM Shield-1 versus mock-treated cells and (3) treatment with 1 μM Shield-1 versus mock-treated cells. Each comparison set was composed of data from six microarrays (three mock-treated and three Shield-treated samples). After selection and filtering, there were 18,257 independent oligo features in the 10 nM Shield-1 set, 19,307 features in the 100 nM Shield-1 set and 13,469 features in the 1 μM Shield-1 set that met the criteria described above. The complete microarray data set is available at the Stanford Microarray Database (http://smd.stanford.edu) and was deposited in the Gene Expression Omnibus database (accession no. GSE5916).

References for Supporting Information

1. Braun, P.D., and Wandless, T.J. (2004) *Biochemistry* **43**, 5406-5413.
2. Tusher, V.G., Tibshirani, R., and Chu G. (2001) *Proc Natl Acad Sci USA* **98**, 5116-5121.